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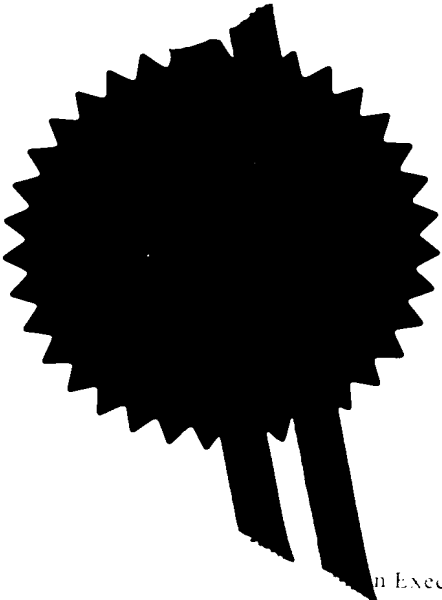Cardiff Road
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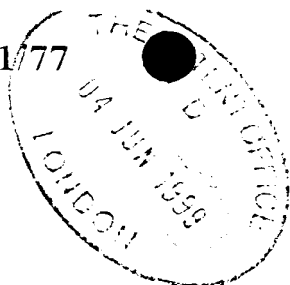
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5. Name of your agent *(if you have one)*   D YOUNG & CO

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METHOD AND APPARATUS FOR ENZYME DETECTION

Field of the Invention

5   The present invention relates to a method for detecting the presence of an enzyme, and an apparatus for use in the method.

Background of the Invention

10

Enzyme electrodes are well known in the art. For example, WO 87/07295 and WO 89/03871 disclose enzyme electrodes capable of responding amperometrically to the catalytic activity of the enzyme in the presence of its respective substrates, wherein the enzyme is immobilised or adsorbed onto the surface of an electrically conductive
15   support member.

The advantages of amperometric biosensors which incorporate enzymes have been reviewed in some detail (Aston and Turner, *Biotech. Genet. Eng. Rev.* 1984, 1, 89-120, ed. G. Russell, Intercept, Newcastle-upon-Tyne; Davis G., *Biosensors*, 1985,
20   1, 161-178). The biosensors discussed therein vary in the mode of signal transduction and are loosely classified as (a) those in which the electrical response arises from the oxidation of a product of the enzyme reaction at an electrode, (b) "mediator assisted" reactions in which the electrons are transported from the enzyme to the electrode with the aid of an oxidation-reduction ("redox") reagent, or
25   (c) "direct electron transfer (DET) in which no such mediator assistance is required.

There are several disadvantages associated with the use of a mediator in signal transduction, including the possibility of the mediator leaching out from the region containing the biocatalyst, diffusion limitations of oxidised and/or reduced forms,
30   and the inherent instability of the mediator itself. As a consequence, mediatorless biosensors have been targeted as an alternative (Tarasevich, *Bioelectrochemistry*, 1985, 10, 231-295).

Ianeillo *et al* (1982) *Anal Chem* 54, 1098-1101, describes mediatorless sensors in which glucose oxidase and L-amino acid oxidase were covalently bonded to a graphite electrode by the cyanuric chloride method. However, it was shown that these enzyme electrodes had only a limited working lifetime (Ianiello and Yacynynch, *Anal Chem* 1981, 53, 2090-2095).

Up to now, mediatorless enzyme electrodes have often incorporated conducting organic polymers, e.g. structural units similar to that of methyl viologen, and/or conducting organic salts such as NMP^+TCNQ^- (N-methyl phenazinium tetracyano-4-quinodimethane) which modify the properties of the electrode and fulfil the role of mediators. However, due to the instability of many conducting polymers, mediatorless electrodes of this type commonly exhibited a short half life and were often oxygen sensitive.

More recently, a novel sensor principle based on measurement of capacitance changes produced during enzyme catalysed dissolution of polymer coatings on electrodes has been developed (McNeil, C.J.; Athey, D.; Ball, M.; Ho, W.O.; Krause, S.; Armstrong, R.D.; Wright, J.D.; Rawson, K., *Anal Chem* 1995, 67, 3928-3935). Electrodes were coated with a biodegradable coating, a copolymer of methyl methacrylate and methacrylic acid. Dissolution is exemplified by a localised increase in pH near the surface of the coating due to the enzymatic reaction between urea and urease. Film degradation is accompanied by an increase in capacitance of up to four orders of magnitude. The method has been developed into a fast and simple disposable sensor for urea in serum and whole blood (Ho, W.O., Krause, S., McNeil, C.J., Pritchard, J.A., Armstrong, R.D., Athey, D. and Rawson, K. 1999 'Electrochemical sensor for measurement of urea and creatinine in serum based on AC impedance measurement of enzyme-catalyzed polymer breakdown'. *Anal Chem* In Press). Furthermore, it has been demonstrated that the high sensitivity and the fast response of this technique could be utilised for immunosensing using urease as the enzyme label.

However, there are several drawbacks associated with the above method, including the time required to produce the localised pH change to dissolve the polymer, the addition of an enzyme substrate and the need to wash (remove) excess enzyme label. Furthermore, following the polymer degradation by capacitance measurements only works effectively if the polymer coating is sufficiently insulating.

Thus, the present invention is advantageous as it addresses the aforementioned problems associated with the prior art.

10

Statements of Invention

In a broad aspect, the present invention provides sensors based on the enzyme-induced degradation of polymer films.

15

In one embodiment of the present invention there is provided a method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer being degraded by said enzyme to produce a signal; and measuring any signal produced.

20

In a preferred embodiment of the present invention the signal is measured by detecting changes in the polymer layer using quartz crystal microbalance.

25

In a second preferred embodiment of the invention the signal is measured by detecting changes in the polymer layer using surface plasmon resonance.

In a third preferred embodiment of the invention the signal is measured by detecting changes in the polymer layer using ellipsometry.

30

In a fourth preferred embodiment of the invention the signal is measured by

detecting changes in the polymer layer using electrochemical impedance spectroscopy.

In a fifth preferred embodiment, the signal is measured by detecting changes in the polymer layer using capacitance measurements.

In one embodiment of the present invention the substrate is an electrode.

In a further embodiment of the invention, the substrate is a capacitor.

In another embodiment of the present invention the substrate is a transducer.

Preferably, the transducer is an electrochemical transducer, an optical transducer or a capacitor.

The enzymes of the present invention may be the analytes present in the sample or introduced as part of reagent system (e.g. an immunoassay label to detect an analyte present in the sample).

In one embodiment of the invention the biodegradable polymer is a poly (ester-amide) and the enzyme is a protease.

In another embodiment of the invention, the biodegradable polymer is a dextran hydrogel and the enzyme is a dextranase.

In another embodiment of the invention, the biodegradable polymer is an albumin crosslinked polyvinylpyrrolidone hydrogel and the enzyme is a pepsin

In another embodiment of the invention, the biodegradable polymer is a polyester such as poly (trimethylene succinate) and the enzyme is a lipase.

In a further embodiment the invention provides an assay comprising the steps of

bringing a sample to be detected for the presence of an analyte into contact with a substrate comprising binding sites for the analyte, in the presence of a conjugate of the analyte and an enzyme label; and detecting the presence of unbound conjugate using the method of the present invention.

5

Preferably the samples used in the current invention are in the form of an aqueous sample, or a biological fluid, for example, blood, urine, serum, plasma or saliva.

In a further aspect, the present invention provides an apparatus for detecting the
10   presence of an enzyme according to the method of any preceding claim comprising a substrate, at least part of which is covered with a biodegradable polymer.

Detailed Description of the Invention
15

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying figures in which:

20

Fig. 1 shows the structure of poly(ester amide).

Fig. 2 shows SPR measurements showing degradation at different concentrations of α-chymotrypsin: (a) 4×10^{-10}M, (b) 4×10^{-9}M, (c) 9.6×10^{-9}M, (d) 2×10^{-8}M, (e)
25   2.8×10^{-8}M, (f) 4×10^{-8}M, (g) 1.2×10^{-7}M, (h) 2.8×10^{-7}M. (where 1 element = 5.3×10^{-3} degrees.)

Fig. 3 shows calibration curves for α-chymotrypsin assay for different molecular weights of poly(ester amide).

30

Fig. 4 shows impedance measurements during degradation of poly (trimethylene succinate) in the presence of lipase.

Fig. 5 shows SPR measurements during degradation of poly(trimethylene succinate) films at different concentrations of lipase from *Pseudomonas fluorescens* (42.5 U/mg).

5

In a preferred embodiment of the present invention, biodegradable polymer films are deposited onto the transducer surface of known thickness and are then dissolved (degraded) directly due to an enzyme or enzyme label acting on the polymer film. The enzyme or enzyme label is in close proximity or attached to the polymer film. 10 The films proposed in the present sensor system are very homogeneous and respond in a matter of minutes due to enzyme amplification, thus resulting in higher sensitivities and lower limits of detection. Coating degradation may be followed using SPR, QCM or ellipsometry and the rate of dissolution of the film has been shown to be directly related to the concentration of enzyme.

15

Thus the present invention provides a new generic sensor format using biodegradable polymers such as poly (ester amides) which can be degraded specifically as a direct result of an enzymatic reaction. The major advantage over the prior art is that the electrode coating itself serves as the enzyme substrate, i.e. 20 no additional enzyme substrate needs to be added or immobilised in order to mediate between the enzymatic reaction and the electrode coating. The present invention simplifies the sensor by reducing the number of sensor components and reactants as well as removing the absolute requirement for additional washing steps in the immunoassay. As a consequence, production costs should be reduced and the 25 sensor systems should display increased reliability.

The signals measured in the present invention may be produced in response to a reduction of the polymer layer on the substrate, either in terms of the area of the substrate covered by the polymer layer, or in terms of the depth of the polymer 30 layer. The signal may also be produced in response to the quality of the polymer layer, for example in terms of pore fromation, swelling and/or delamination.

Quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and ellipsometry may be used to determine properties of surfaces and thin films. All of these techniques have been applied successfully to biosensing, especially to monitoring of direct binding events between antigens and antibodies (Rickert, J,;

5   Brecht, A.; Gopel, W., *Biosensors and Bioelectronics* 1997, 12, 567-575: Toyama, S.; Shoji, A.; Yoshida, Y.; Yamauchi, S.; Ikariyama, Y., Sensors and Actuators B-Chemical 1998, 52, 65-71: Arwin, H., *Thin Solid Films* 1998, 313-314, 764-774). Direct binding produces changes to the electrode surfaces that are more indicative of a porous layer, resulting in very small changes being observed.

10

Electrochemical impedance spectroscopy provides information about film properties such as incomplete coverage, pore formation, swelling and delamination. The initial film quality and film degradation of the present invention may be studied using electrochemical impedance spectroscopy over a frequency range from 0.1mHz to

15   100kHz. In addition to the information extracted from impedance spectroscopy, impedance measurements at quartz crystals provide data such as changes in mass and the visco-elastic properties of the films during degradation. In order to extract this information, the quartz-crystal impedance spectra may be fitted to the equivalent circuit of a coated quartz crystal given in Auge, J.; Hauptmann, P.;

20   Eichelbaum, F.; Rosler, S., *Sensors and Actuators B-Chemical* 1994, 19, 518-522. Typically, impedance measurements are performed at polymer coated quartz crystals at a number of frequencies close to the resonance frequency of 10 MHz.

The term degradation is used in its conventional sense, i.e., a chemical reaction in

25   which a compound is converted, or decomposes in some way, to give a simpler compound, for example, by dissolution. Monitoring film degradation using QCM, SPR, ellipsometry or electrochemical impedance spectroscopy has shown that the rate of dissolution of the polymer film is directly related to the enzyme concentration.   Thus, using this system for the development of a generic

30   immunosensor with enzyme as the antigen and antibody label has the advantage that no enzyme substrate needs to be added. The electrode coating itself serves as the substrate thus making the electrode an integral part of the sensing process and

eliminating washing steps otherwise required in standard immunoassay techniques.

The electrodes of the present invention are noble metal electrodes. Noble metals include metals such as gold, silver and platinum, or alloys thereof, which display
5   resistance to corrosion or oxidation. Preferably the electrode is gold. Typically, the gold is deposited by thermal evaporation onto a chromium coated glass slide. The thickness of the gold coating may vary considerably, but is usually between 20 and 100 nm. Preferably, the thickness of the gold coating is between 45 and 80 nm.

10   The substrate is coated with a film of the biodegradable material. The film may range from monolayers to several hundred nm thick. Preferably, the film is from 5 to 100 nm thick. More preferably, the film is 10 to 100 nm thick. Typically, the films are deposited on the surface of the substrate by spin-coating using, a solution of the polymer in an appropriate solvent (for example, chloroform, or acetone).
15   The biodegradable films degrade rapidly under the catalytic action of a specific enzyme directed to the polymer used to coat the substrate.

The preferred coatings in the present application degrade within a matter of seconds or minutes in the presence of a single enzyme, thereby leading to a fast sensor
20   response. In contrast, up to now, most biodegradable materials described in the literature are reported to degrade over the course of several hours or days (Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R., *Macromolecular Chemistry and Physics* 1994, 195, 2279-2289; Brondsted, H.; Hovgaard, L.; Simonsen, L., *Stp Pharma Sciences*
25   1995, 5, 60-64). Thus the present invention provides a sensor displaying short response times.

The assay of the present invention typically employs binding pairs. A non-exclusive list of commonly used binding pairs includes avidin/biotin, antibody/antigen,
30   haptens and nucleic acid (DNA and RNA). Generally, when the binding pair is antibody/antigen the assay is referred to as an immunonassay. Other biosubstances capable of molecular recognition include lectins for saccharides, hormone receptors

for hormones and drug receptors for drugs and active drug metabolites.

In a preferred aspect, the method of the present invention is used for performing an immunoassay.

Typically, in enzyme immunoassays, an enzyme is used as a label or marker which is bound to one member of the antigen-anotibody pair identical to that in the sample to be measured. The enzyme bound antigen/antibody then competes with the sample antigen/antibody for the binding site on a limited supply of its complement antibody/antigen.

Classical methods for immunoassay include:

(i) a capture antibody on a solid phase, such as a plastic microtitre plate, exposure to the biological sample to attach the antigen of interest, washing and then exposure to a second labelled antibody. The label on the antibody may be an enzyme for example. Further washing is followed by detection of the label (and hence the amount of antigen in the original sample). This is known as a sandwich assay or two-site assay.

(ii) a capture antibody on the solid phase followed by exposure to the biological sample containing antigen and an added amount of labelled antigen. Labelled and unlabelled antigen compete on the solid phase for the antibody sites. The amount of label revealed after washing is inversely proportional to the amount of true antigen in the biological sample. This is known as a competitive assay.

The concept of integrating enzyme and immunoassay techniques into the sensor devices disclosed in the present invention thus offers the prospect of reagentless analysis with little or no sample preparation. The major advantage of this approach for medical use is ease of operation, thereby allowing deployment of sensors in decentralised laboratories and facilitating a more rapid return of clinical information. The net benefit is an earlier institution of appropriate therapy.

In a preferred embodiment an immunosensor can be produced where the sample

flows through a series of zones. The first of these is a blood separation membrane, which removes the cellular component. In the next zone, the capture antibody or antigen is immobilised on a substrate such as nitrocellulose membrane or polystyrene. A sample is introduced containing the analyte to be measured and mixes with an enzyme/antigen or enzyme/antibody conjugate. The mixture of analyte and conjugate will then flow over the capture antibody or antigen. Both conjugate and analyte compete for the binding sites. Flow through the capture membrane will remove some of the enzyme-analyte conjugate in a competitive manner. In the next zone, the unbound complex reaches the biodegradable polymer and causes it to degrade. The rate of polymer dissolution is directly proportional to the amount of analyte in the sample. The immunoassay can be set up in the competitive or sandwich assay format.

The present invention is further illustrated by way of the following non-limiting examples.

Example 1

One such biodegradable material is poly (ester amide) shown in Figure 1. Poly (ester amides) of 18800 g mol^{-1}, 10218 g mol^{-1} and 6407 g mol^{-1} were synthesised by polycondensation as described in Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.; Katsarava, R., *Macromolecular Chemistry and Physics* 1994, 195, 2279-2289. The polydispersity of the polymer was lowered to 1.28 using fractionation. 3 nm chromium and then 45 nm gold were thermally evaporated onto the whole surface of the glass slides. The shape is not critical as the laser beam hits the middle of the surface. The gold layer is on top of the chromium and therefore always in contact with the polymer. The two metals were deposited without opening the evaporator in between. 10 nm thick films of the poly (ester amides) were spin-coated onto the gold surface from a 0.13 w% solution of the polymer in chloroform at a speed of 3000 rpm. The polymer films were left to dry at room temperature for at least 24 hours.

Electrochemical impedance spectroscopy and SPR were used to characterise the

polymer films and to follow their degradation in the presence of α-chymotrypsin. The cell was thermostated at 25°C to eliminate any effect that temperature changes may cause in the rate of the polymer degradation. The detection system of the SPR monitor essentially consists of a monochromatic and polarised light source, a glass

5   prism, a thin metal film in contact with the prism, and a photodetector.

The polymer was degraded rapidly by the proteolytic enzyme α-chymotrypsin. The rate of hydrolysis of esters is $\sim 10^5$ times higher than the corresponding amides when both are catalysed by α-chymotrypsin (Arabuli, N.; Tsitlanadze, G.; Edilashvili, L.; Kharadze, D.; Goguadze, T.; Beridze, V.; Gomurashvili, Z.;

10   Katsarava, R., *Macromolecular Chemistry and Physics* 1994, 195, 2279-2289), i.e. α-chymotrypsin preferentially attacks the ester bonds. α-Chymotrypsin is also a suitable enzyme label for immunosensing since it is virtually never present in the blood circulation.

15   The polymer films were shown to be stable in a pH 7.3 buffer containing 140 mM NaCl and 10 mM phosphate. Addition of α-chymotrypsin to the buffer solution resulted in rapid and reproducible polymer breakdown. The degradation of the polymer was complete in less than 20 minutes for enzyme concentrations greater than 9×10^{-9} mol/l (see Figure 2). After an initial period the SPR response changed

20   linearly with time. To obtain a calibration graph, the slopes of the linear region of the breakdown curves in Figure 2 were calculated and the data presented in Figure 3. Using the rate of change as a measure for the enzyme concentration rather than the time needed to degrade the film completely has the advantage, that considerably lower enzyme concentrations can be detected in a reasonable period of time.

25   However, to visualise the sensitivity over the whole concentration range, a logarithmic plot is shown (Figure 3). The calibration graph (Figure 3) shows that α-chymotrypsin concentrations as low as 4×10^{-10} mol/l could be detected in less than 30 minutes. The degradation was shown to be dependent on the enzyme concentration. Thus, the system can be used as part of an immunosensor based on

30   the detection of α-chymotrypsin concentrations.

Example 2

Polyesters such as poly (trimethylene succinate) can be hydrolysed by lipases. The dissolution of poly (trimethylene succinate) powder and films was investigated by Walter, T.; Augusta, J.; Muller, R.J.; Widdecke, H.; Klein, J., Enzyme and Microbial Technology 1995, 17, 218-224. The enzyme activity for the interaction of lipase with an insoluble substrate was found to be highly reproducible. Succinic acid (38.8g, 0.33mol) and 1,3-propanediol (26.25mL, 0.33mol) were mixed in a flask under nitrogen with mechanical stirring and heated slowly to 90°C. Methane sulphonic acid (0.071g, 0.07mmol) was then added and the temperature raised to 100°C. Water vapour was evolved and collected using a microdistillation head. The reaction was left overnight at 100°C then cooled to give a thick orange oil. Gel Permeation Chromatography (GPC) showed that the molecular weight was still very low so 28.0g of the pale orange oil was further reacted with (0.10g, 1mmol) of the catalyst overnight at around 100°C, the temperature was then increased to 140°C to remove any excess water. The polymer was then isolated by precipitation from methanol overnight to give a high molecular weight fraction of polymer. Total yield was 3g. GPC - Mw = 5765, Polydispersity = 1.212

Glass substrates were prepared from microscope slides and washed by boiling in 50:50 nitric acid (70%): hydrogen peroxide for 5 minutes. The slides were then washed with ultra pure water and dust free methanol before blow-drying with nitrogen. Chromium was then deposited to give a ~20nm layer, followed by an ~80nm layer of gold. Vacuum deposition was carried out with an Edwards E306A coating system, and an IL150 quartz crystal rate monitor was used to monitor the deposition rate and layer thickness. The gold-coated slides were spin-coated with the poly (trimethylene succinate) using acetone as the solvent and a concentration of 0.093g/mL of the polymer. As previously the electrodes were dried for 24h at room temperature before use. All the impedance measurements were performed using an Autolab frequency response analyser. Measurements were conducted at zero potential using a 2-electrode system with a platinum electrode as the counter electrode in parallel to the polymer coated gold electrode The polymer coated metallised glass slides were placed into the bottom of a Perspex well. The

dimensions of the measuring area were determined by an O-ring with an inner diameter of 7 mm which formed the waterproof connection between the metallised glass slide and the well. A platinum coil was placed opposite the working electrode at a distance of ~ 4 mm and served as the counter electrode. The electrodes were characterised in 0.5mL of a pH 7.4 buffer solution containing 10mM phosphate and 100mM sodium chloride. The effect of the enzyme addition was investigated. Measurements were performed at a single frequency (3.5kHz) every 10s. 0.5mL of lipase solution (1mg/ml of lipase from *Pseudomonas fluorescens* with an activity of 42.5 U/mg in buffer) was added after around 20 min. The result (Figure 4) shows polymer degradation.

The degradation of poly (trimethylene succinate) films in the presence of different concentrations of lipase was also followed using SPR measurements (Figure 5). Substrate preparation, spin coating and SPR measurements were carried out under the same conditions as described in Example 1.

Claims

1.   A method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer being degraded by said enzyme to produce a signal; and measuring any signal produced.

2.   A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using quartz crystal microbalance.

3.   A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using surface plasmon resonance.

4.   A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using ellipsometry.

5.   A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using electrochemical impedance spectroscopy.

6.   A method according to claim 1 wherein the signal is measured by detecting changes in the polymer layer using capacitance measurements.

7.   A method according to claim 1 wherein the substrate is a transducer.

8.   A method according to claim 7 wherein the substrate is an electrode.

9.   A method according to claim 7 wherein the substrate is a capacitor.

10.   A method according to claim 7 wherein the transducer is an electrochemical transducer or an optical transducer.

11.   A method according to any preceding claim wherein the biodegradable polymer is a poly (ester-amide) and the enzyme is a protease.

12.   A method according to any one of claims 1 to 10 wherein the biodegradable polymer is a dextran hydrogel and the enzyme is a dextranase.

13.   A method according to any one of claims 1 to 10 wherein the biodegradable polymer is an albumin crosslinked polyvinylpyrrolidone hydrogel and the enzyme is a pepsin.

14.   A method according to any one of claims 1 to 10 wherein the biodegradable polymer is a polyester such as poly (trimethylene succinate) and the enzyme is a lipase.

15.   An assay comprising the steps of bringing a sample to be detected for the presence of an analyte into contact with a substrate comprising binding sites for the analyte, in the presence of a conjugate of the analyte and an enzyme label; and detecting the presence of unbound conjugate using the method of any one of claims 1 to 14.

16.   A method according to any preceding claim wherein the sample is an aqueous sample, or a biological fluid.

17.   An apparatus for detecting the presence of an enzyme according to the method of any preceding claim comprising a substrate, at least part of which is covered with a biodegradable polymer.

ABSTRACT

METHOD AND APPARATUS FOR ENZYME DETECTION

5   The present invention provides a method for detecting the presence of an enzyme based on the enzyme-induced degradation of a polymer film. In a preferred embodiment, the invention provides a method for detecting the presence of an enzyme comprising contacting the sample to be analysed with a substrate, at least part of which is covered with a layer of a biodegradable polymer, said polymer

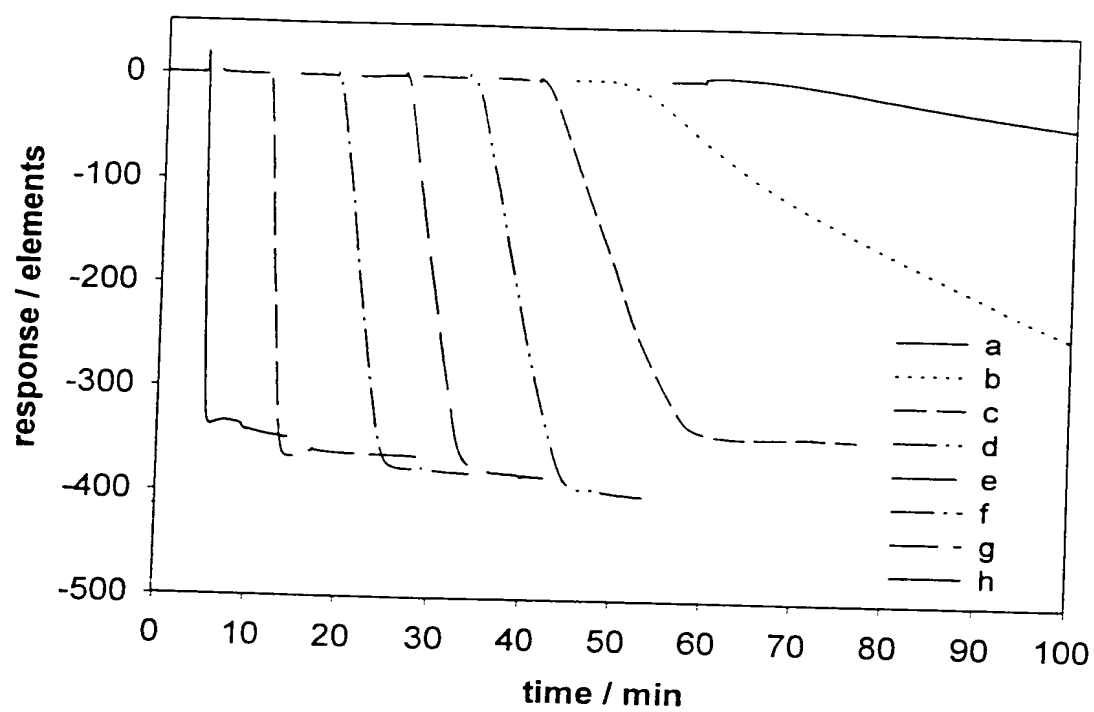10   being degraded by said enzyme to produce a signal; and measuring any signal produced.
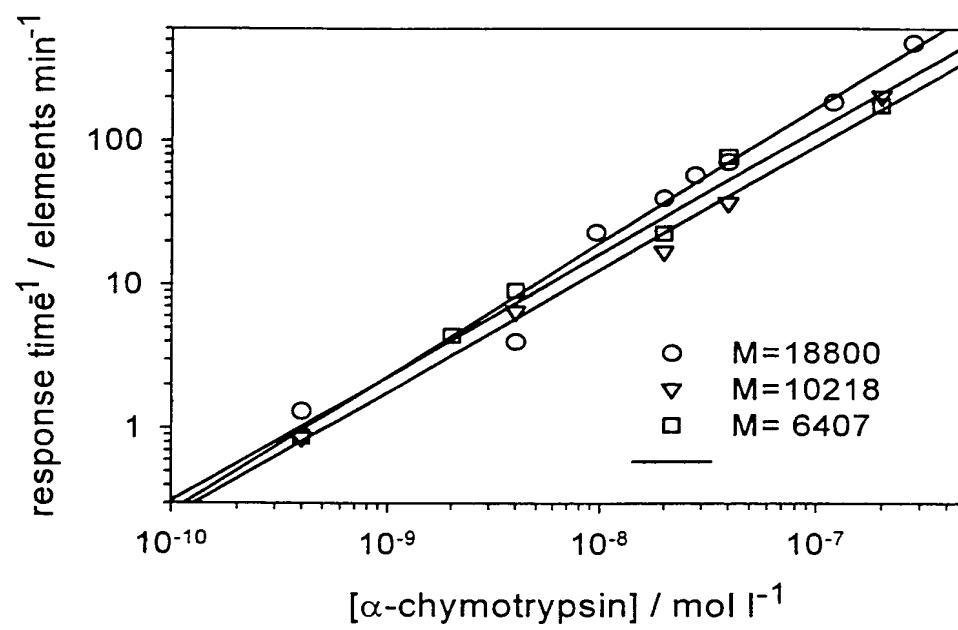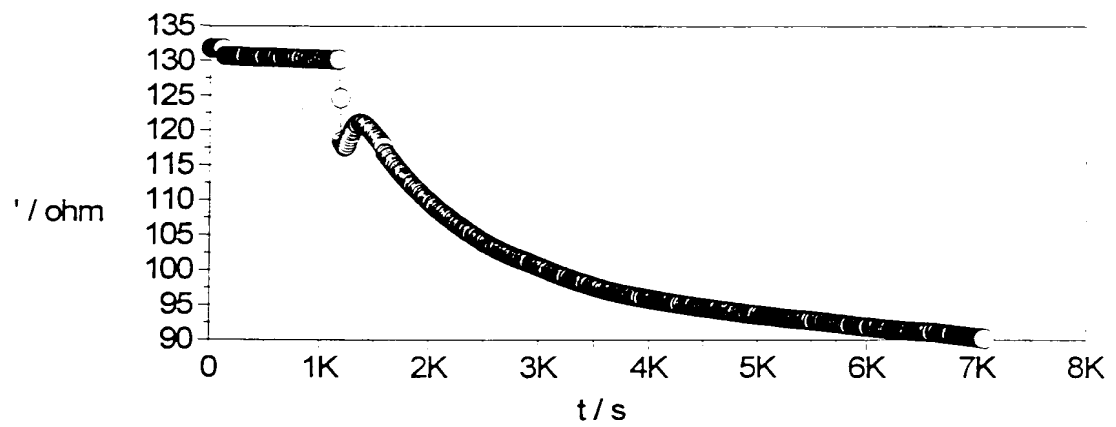
Figure 1

Figure 2

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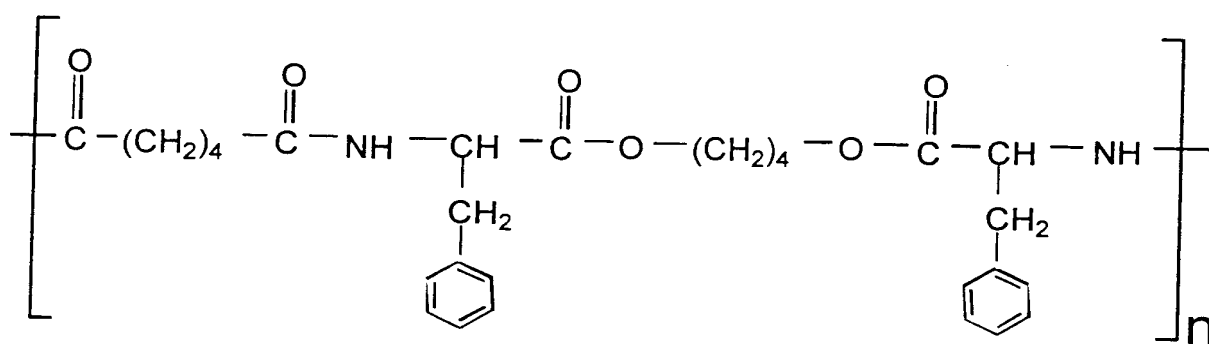
Figure 3

Figure 4

Figure 5

Figure 5